Information Disclosure Statement and copies of the references cited therein. These references are submitted with regard to the rejection of the claims under 35 USC § 101. Applicants contend that these references corroborate applicants' position relating to the utility of the Stat3 protein of the invention.

Reconsideration of the claims is requested in view of the following remarks.

**Rejections Under 35 USC § 101**

Claims 1, 97, and 108-113, and 115-119 have been rejected under 35 USC § 101 because the claimed invention is allegedly not supported by either a specific asserted utility or a well-established utility. Applicants strenuously disagree with the Examiner's position.

As presented throughout the specification, Stat3 proteins are useful for a variety of purposes. To begin, it should be evident that amino acid sequences of Stat3 protein may be used to generate antibodies. See, for example, page 13, lines 7-15. Indeed, this utility is demonstrated by the generation of Stat3 specific antibodies. See page 72, line 22 over to page 73, line 11. The Examiner appears to appreciate the proof of this utility, as the Examiner acknowledges that antibodies which are immunologically specific for SEQ ID NO: 12 have been made by the present inventors.

Additional evidence is presented throughout the specification that Stat3 was known to be involved in a number of intracellular signaling pathways, including those triggered in response to epidermal growth factor (EGF) and interleukin-6 (IL-6). See, for example, page 71, lines 6-8. Corroborating evidence for this statement is presented in the scientific literature of the time, a subset of which are presented herein for the Examiner's consideration and included in a Supplemental Information Disclosure attached hereto. See, for example, Zhong et al. (1994) Science 264:95-98.

Indeed, by 1997, Stat3 had been shown to be critical for IL-6- and IL-6 family member-mediated biological effects in numerous studies. Moreover, Stat3 had also been shown to be activated in response to a variety of cytokines, including granulocyte colony-stimulating factor (G-CSF) and EGF. See Takeda et al. (1997) PNAS 94:3801-3804; e.g., page 3801, left column, bottom paragraph and references cited therein. The Takeda et al. reference also demonstrated that Stat3 activity is essential for early embryonic development.

By 1999, the scientific literature also recognized the contribution of Stat3 to interferon (IFN) signaling pathways. Specifically, Stat3 had been shown to be a component of the IFN-beta signaling pathway and, significantly, Stat3 over-expression in IFN resistant cells restored IFN-mediated antiviral and antiproliferative responses in these cells. See Rani et al. (1999, J Biol Chem 274:32507-32511) and references cited therein. In view of the above, it is apparent that Stat3 was known to be an important, if not essential, component of a variety of signaling pathways at the time the instant application was filed. Applicants assert that the biological role(s) of Stat3 in the EGF, IL-6, IFN signaling pathways and the acknowledged importance of these pathways in, for example, cellular proliferation, transformation, inflammation, and immune response to viruses would render perfectly apparent multiple applications for which the amino acid sequence of Stat3 would be useful.

The instant application also describes the utility of the receptor recognition factor(s) of the invention, of which Stat3 is a representative member, for both diagnostic and therapeutic applications. The present invention encompasses the use of Stat3 protein (or its chemical or pharmaceutical cognates, analogs, or fragments), for example, as a modulator of the activity initiated by the stimulus bound to a receptor for which Stat3 is a downstream signaling molecule. For applications in which the activity initiated by the stimulus bound to a receptor is insufficient, the introduction of the Stat3 protein or its chemical or pharmaceutical cognates, analogs, or fragments is envisioned. For applications in which it is desirable to reduce the activity initiated by the stimulus bound to a receptor, the introduction of a Stat3 inhibitor is envisioned. See page 38, line 21 over to page 39, line 8. Such a Stat3 inhibitor may be, for example, a mutant Stat3 protein or a Stat3 fragment, either of which is capable of competing with the full length functional protein for binding to components of the signaling pathway, but are at least partially impaired with regard to function. See, for example, page 12, lines 1-11. Such Stat3 inhibitors may be referred to as Stat3 dominant negative proteins. See below for evidence presented in the literature of the utility of such Stat3 dominant negative proteins/molecules.

Moreover, a body of scientific literature was available prior to the filing date of the instant application which clearly supports Applicants' contention that Stat3 proteins were known to participate in a variety of signaling pathways implicated in various diseases/disorders and were, therefore, of utility with regard to diagnostic and therapeutic applications pertaining to

these diseases/disorders. By 1999, Stat3 had been shown to be required for oncogenic transformation by Src. See, for example, Turkson et al. (1998) Molecular and Cellular Biol 18:2545-2552 and Turkson et al. (1999) Molecular and Cellular Biol 19:7519-7528, and references cited therein. It is noteworthy that a number of studies had provided evidence that the aberrant activation of Stat3 signaling was implicated in the malignant progression of a number of human cancers, including: human breast carcinoma, multiple myeloma, lymphomas, leukemias, and head and neck carcinoma. In the human myeloma cell line, for example, IL-6-mediated constitutive activation of Stat3 signaling induces anti-apoptotic regulators, whereas inhibition of Stat3 signaling results in a dramatic induction of programmed cell death (apoptosis) of these transformed cells. These findings provided evidence that aberrant Stat3 signaling contributes to malignant progression of multiple myeloma by inhibiting apoptosis. See Niu et al. (1999) Cancer Res 59:5059-5063, e.g., page 5059, left column, bottom paragraph through right column, second paragraph and references cited therein. The results presented by Niu et al. also demonstrate that inhibition of activated Stat3 signaling can also suppress the growth of a melanoma cell line and induce apoptosis of these tumor cells *in vivo*. The contribution of Stat3 to drug resistance in multiple myeloma was also described by Dalton and Jove, wherein the authors suggest that agents which serve to down-regulate the activity of Stat3 (i.e., a Stat3 dominant negative molecule) could be used to enhance sensitivity to chemotherapy and/or prevent the development of resistance. See Dalton and Jove (1999) Semin Oncol 26:23-27, abstract.

Significantly, support for the utility of the molecules of the invention in the treatment of various cancers is presented in the instant specification. See, for example, page 15, lines 7-10 and page 16, lines 6-11. Also presented therein is support for the utility of the molecules of the invention in conjunction with IFN-based antiviral therapy. See, for example, page 16, lines 11-13. In view of the above, applicants assert that the teaching set forth in the instant application, in combination with the scientific literature at the time of filing, clearly establishes both specific and substantial utility for the polypeptide of SEQ ID NO: 12 and fragments thereof.

The scientific literature at the time of filing also supports a role for the utility of Stat3 molecules in cardiac remodeling in response to various *in vivo* stimuli. Specifically, Stat3 was shown to contribute to signaling pathways involved in cardiac myocyte hypertrophy (also known

as myocardial hypertrophy), which is induced by *in vivo* stimuli, such as pressure or volume overload. Of note, these signaling pathways were triggered in response to IL-6 and IL-6-like cytokines, such as leukemia inhibitory factor (LIF). See Kodama et al. (1997) Circulation Res 81:656-663, Kunisada et al. (1998) Circulation 98:346-352, and references cited therein. In view of the above, applicants assert that the specification and the scientific literature published at the time of filing underscore the utility of Stat3 for evaluating and/or modulating conditions relating to IL-6 and IL-6-like cytokine signaling, such as conditions relating to myocardial hypertrophy.

Applicants submit that when the teachings of the specification are combined with the knowledge in the art at the time the application was filed, the activation of Stat3 by EGF, IL-6, IFN-beta, would be recognized as useful by one of skill in the art as providing a method for modulating the effects of the cytokines and growth factors. In view of the above, applicants have presented a preponderance of evidence attesting to a plurality of specific and substantial utilities for Stat3 (SEQ ID NO: 12).

In light of the above remarks, Applicants submit that the rejection under § 101 is inappropriate and respectfully request that this rejection be withdrawn.

**Rejection Under 35 USC § 112, First Paragraph**

Claims 1, 97 and 108-113 and 115-119 have been rejected under 35 USC § 112, first paragraph, as allegedly not enabled on the basis that the claimed invention is not supported by either a specific or substantial utility. This rejection is respectfully traversed. The above remarks responsive to the rejection under § 101 are fully applicable to this rejection and are herein specifically incorporated by reference.

As described hereinabove, the present invention encompasses the use of Stat3 protein, chemical or pharmaceutical cognates thereof, analogs thereof, mutants thereof, or fragments thereof, for example, as a modulator of the activity initiated by the stimulus bound to a receptor for which Stat3 is a downstream signaling molecule. As described in the specification, the term modulator may refer, for example, to a Stat3 protein, Stat3 mutant protein, or a fragment, which is capable of either increasing or decreasing an activity initiated by the stimulus bound to a receptor for which Stat3 is a downstream signaling molecule. See page 38, line 21 over to page 39, line 8. A skilled artisan reading the present specification would appreciate that Stat3

proteins, functional fragments thereof, analogs, and/or cognates, for example, could be used to increase an activity initiated by the stimulus bound to a receptor for which Stat3 is a downstream signaling molecule. Support for the above is presented throughout the specification.

Moreover, the specification presents ample guidance pertaining to making and using the above Stat3 proteins and related molecules. A skilled artisan, having read the specification would also appreciate that a mutant Stat3 protein or a Stat3 fragment, for example, either of which are capable of competing with the full length functional protein for binding to components of the signaling pathway, but are at least partially impaired with regard to function, could be used to decrease an activity initiated by the stimulus bound to a receptor for which Stat3 is a downstream signaling molecule. In a particular embodiment of the invention, such Stat3 antagonists are described as a peptide having the sequence of a portion of an SH2 domain of a STAT protein (e.g., Stat3) or the phosphotyrosine domain of a STAT protein (e.g., Stat3), or both. Such peptides or fragments would inhibit Stat3 signaling, for example, by disrupting dimerization of Stat3, which is required for functional activity. See page 12, lines 1-11. Such Stat3 inhibitors may be referred to as Stat3 dominant negative proteins. Moreover, a skilled artisan would be familiar with the scientific literature regarding Stat3 and would, therefore, be aware of the Stat3 dominant negative protein described by Dalton and Jove (1999) Semin Oncol 26:23-27. In view of the above, applicants submit that the rejection under 35 USC § 112 for an alleged lack of enablement is improper and should be withdrawn.

Claims 111-113 are further rejected under 35 USC § 112 for an alleged lack of enablement because they are drawn to an immunogenic fragment of SEQ ID NO:12. It is the Examiner's position that the specification does not provide guidance as to which regions of SEQ ID NO:12 could be used as immunogenic fragments. The term "immunogenic fragment" is a well known term in the art. As used in the instant application and understood by the ordinarily skilled artisan, the term refers to those fragments of a molecule which are likely to elicit an immunological response.

A skilled artisan, having read the application which includes the amino acid sequence of Stat3 (SEQ ID NO:12), would readily be able to identify immunogenic fragments using a variety of basic computer programs such as those based on, e.g., a Kyte/Doolittle analysis. Such programs are based on an assessment of the hydrophobicity of different regions of a protein. In

general, and as well known in the art, the more hydrophilic an amino acid region of a protein, the more likely it is to be antigenic. Such programs are regularly utilized in laboratories specializing in the biological sciences and can be accessed from a number of electronic database sources. Notably, a number of companies that specialize in the generation of antibodies routinely perform analyses of amino acid sequences to determine the immunogenicity of various subportions (i.e., fragments) of a larger protein. Indeed, the availability of such services underscores the routine nature of the analysis.

A skilled artisan, having the amino acid sequence of SEQ ID NO: 12 would also appreciate that an analysis of the sequence to identify domains of known function would also be useful in the identification of immunogenic fragments. Relatedly, the STAT molecules were known to share a number of common regions or domains, including: an SH3 domain, and an SH2 domain, and recognition sites for tyrosine and serine/threonine kinases. See Darnell (1997) Science 277:1630-1635. A skilled artisan would, therefore, appreciate which of these domains had been demonstrated to be immunogenic in other proteins and use this information to advantage in the determination of immunogenic fragments in the context of Stat3.

In addition to the programs that analyze the primary structure (i.e., the amino acid sequence) of a protein, there are computer programs capable of predicting the three-dimensional structure of a protein based on the amino acid sequence. Such programs may be used to predict which amino acid sequences are located on exposed regions of a protein and, therefore, would be good candidates for immunogenic fragments. An ordinarily skilled practitioner in the art would be aware of all of the above-mentioned programs and resources available for determining the identity of immunogenic fragments comprised in an amino acid sequence.

Moreover, guidance pertaining to the choice of immunogenic fragments is provided in a variety of laboratory manuals routinely available in biological laboratories. See, for example, Antibodies: A Laboratory Manual, Harlow and Lane, eds. Cold Spring Harbor Laboratory, New York (1988), which was incorporated into the instant application by reference at page 41, lines 12-14. Chapter 5, Index pages and pages 72-77 of this reference are submitted herein as Exhibit A. Considerations regarding the choice of an appropriate amino acid sequence or sequences are described in detail therein. Such considerations include, for example, the length of the amino acid sequence and the hydrophobicity of an amino acid region, wherein hydrophilic amino acid

stretches are preferred immunogenic fragments. The following approach is generally recommended for generating antibodies immunospecific for a particular protein: the use of more than one amino acid sequence (peptide fragment); the use of the carboxyl-terminal sequence, particularly if the amino acid sequence is hydrophilic; the use of the amino-terminal sequence, particularly if the amino acid sequence is hydrophilic; and the use of internal hydrophilic regions, preferably comprised of longer peptide fragments. See Exhibit A, page 76, second paragraph. Applicants submit that such disclosures render the generation of antibodies to a known amino acid sequence routine and maintain that a skilled practitioner would be able to practice the invention as claimed.

In view of the above, applicants assert that the rejection of claims 111-113 under 35 USC § 112 is improper. Accordingly, it is respectfully requested that this rejection be withdrawn.

Claims 111-113 have also been rejected under 35 USC § 112 for an alleged lack of written description. The Examiner contends that the specification fails to adequately describe the fragments of SEQ ID NO:12 that would be immunogenic. The arguments set forth above with regard to issues related to enablement are equally applicable with regard to written description. A skilled artisan would appreciate that identification of immunogenic fragments of a protein having a known sequence, such as that of SEQ ID NO: 12, is routine. Indeed, the amino acid sequence of SEQ ID NO: 12 provides ample written description with which a skilled artisan could identify an immunogenic fragment.

In view of the above, applicant believes that the Examiner's objections based on enablement and written description are improper, and withdrawal of this ground of rejection is believed to be in order and is requested.

**Fees**

No fees are believed to be necessitated by the instant response. However, should this be in error, authorization is hereby given to charge Deposit Account no. 11-1153 for any underpayment, or to credit any overpayments.

**Conclusion**

Applicant submits that the claims are in condition for allowance, and accordingly entry of the present remarks, reconsideration and withdrawal of the outstanding grounds of rejection, and

early allowance of the claims are believed to be in order and are courteously solicited.

Applicant respectfully solicits allowance of the pending claims.

Respectfully submitted,

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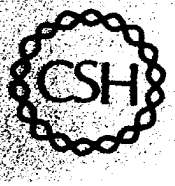
Enclosures: Supplemental IDS and references cited therein
Exhibit A

Qiao-wen Xie

# Antibodies
## A LABORATORY MANUAL

Ed Harlow
Cold Spring Harbor Laboratory

David Lane
Imperial Cancer Research Fund Laboratories




**Cold Spring Harbor Laboratory**
**1988**

# 5

## Haptens

Many small chemicals can be used to raise antibodies, if they are coupled to larger protein molecules. The small compounds are known as haptens, while the proteins to which they are coupled to are called carriers. The haptens themselves serve as epitopes for binding to the antibodies on the B-cell surface, and the carriers provide the class II–T-cell receptor binding sites. In general, haptens should be coupled to soluble carriers such as bovine serum albumin (BSA) or keyhole limpet hemacyanin (KLH). The coupling mechanism will vary with each hapten, but many of the bifunctional coupling reagents listed in Table 5.5 (p. 130) will be helpful. Also, the techniques on the coupling of synthetic peptides to carriers on p. 78 may be applied. In general, approximately 1 mole of hapten per 50 amino acids of carrier is a reasonable coupling ratio.

## Synthetic Peptides

The use of synthetic peptides as immunogens has been an important technique in the elucidation of the properties of an antibody response (e.g., Goebel 1938; Anderer 1963; Anderer and Schlumberger 1965; Sela 1966, 1969; Arnon et al. 1971). Recently, as more DNA sequences

### Choosing between Bacterial Expression and Peptides for Immunogen Production

When a cloned DNA sequence in available, antibodies can be prepared either using peptides or bacterially expressed proteins. There are strong proponents for both approaches, both groups citing their experiences favoring one method over the other. Both have advantages and disadvantages, and for a particular antigen one may be better suited than another. However, if both approaches are available to a researcher, both should be used.

For anti-peptide antibodies, a good response to the desired peptide usually can be generated with careful selection of the sequence and coupling method. Because of the way in which the peptide is displayed to the immune system, most peptides elicit a good response. Therefore, the anti-peptide approach has major advantages if the antigen is known to be highly conserved. Likewise, if antibodies need to be raised against a particular region, anti-peptide antibodies have many advantages. The major disadvantage with anti-peptide antibodies is that they may not recognize the native antigen. The percentage of antibodies raised against peptides that will bind to the native protein will vary from antigen to antigen. Values reported in the

and their corresponding protein sequences have become known, synthetic peptides have been used to prepare antibodies specific for previously uncharacterized proteins (Sutcliffe et al. 1980; Walter et al. 1980; and reviewed in Lerner 1982, 1984; Walter 1986; Doolittle 1976; and in Ciba Foundation 1986). Peptides are normally synthesized using the solid-phase techniques pioneered by Merrifield (1963). The synthetic peptides are purified and coupled to carrier proteins, and these conjugates are then used to immunize animals. In these cases, the peptides serve as haptens with the carrier proteins, providing good sites for class II–T-cell receptor binding. Peptide–carrier conjugates seldom fail to elicit a response because of tolerance. Consequently, the peptides can usually be seen as epitopes, and high-titered antisera commonly are prepared. Characteristically, these antibodies will bind well to denatured proteins, but may or may not recognize the native protein.

The two most important advantages of anti-peptide antibodies are that they can be prepared immediately after determining the amino acid sequence of a protein (either from protein sequencing or from DNA sequencing) and that particular regions of a protein can be targeted specifically for antibody production. Rapid conversion from DNA sequence information to antibodies has enormous potential for application in molecular biology. Similarly, the production of site-specific antibodies has immediate implications for functional and clinical studies.

literature range from 0/4 to 3/4 of anti-peptide antibodies will bind to the native antigen. Synthetic peptide antigens are also more expensive to produce than bacterial fusion protein antigens.

Bacterially expressed antigens present a different set of problems. Some will be difficult to express in *E. coli*, presumably because of their toxic side effects. In these cases, inducible systems such as the T7 systems of Studier (see Rosenberg 1987; Studier and Moffatt 1986) are recommended. Even when high levels of the antigen of interest can be produced, there may be some instances where the protein will not be immunogenic or where the antibodies will not recognize the native protein. However, because of the larger size of the bacterially expressed protein, there is a better chance that the antibodies will bind to the native protein.

A reasonable compromise for antibody production would be: (1) If the budget is limited and/or antibodies for the native protein are essential, use fusion proteins or full-length expression in *E. coli*. (2) If the budget is large enough, try both bacterially produced immunogens and peptides. (3) If the protein is highly conserved, use peptides. (4) If site-directed antibodies are needed, use peptides or prepare large banks of monoclonal antibodies against the bacterially produced immunogens.

The major problem that is encountered when preparing anti-peptide antibodies is whether they will recognize the native protein. Assays that need or benefit from anti-native antibodies, such as immunoprecipitation, many cell staining techniques, or immunoaffinity purification, will succeed only when the peptide sequence is displayed on the surface of the native molecule in a conformation similar to the peptide–carrier conjugate. Therefore, the successful production of anti-peptide antibodies is often determined by the researcher's ability to predict the location of certain peptide sequences in the three-dimensional structure of the protein.

Because of their size, peptides may not be immunogenic on their own. To elicit an antibody response directly, they must contain all of the features of any immunogen, notably they must have an epitope for B-cell binding and a site for class II–T-cell receptor binding. Some peptides, even surprisingly small ones, contain both these sites (or more properly, one sequence that can serve both functions), and these peptides can be used without carriers (e.g., see Beachy et al. 1981; Lerner et al. 1981; Dreesman 1982; Jackson 1982; Atassi and Webster 1983; Young et al. 1983). Unfortunately, there are no methods, short of immunization, to test this, and therefore, most peptides are coupled to carrier proteins before injection. An exciting recent development is the use of synthetic class II–T-cell receptor sites synthesized directly with the desired epitope (Francis et al. 1987; see also, Good et al. 1987; Borras-Cuesta et al. 1987; Leclerc et al. 1987). Although there are not enough cases to determine how widely applicable this approach will be, the concept is provocative. With this strategy, the peptide of interest is synthesized as either an amino- or carboxy-terminal extension of a known class II–T-cell receptor site. The synthetic peptide, now containing both sites, is injected without coupling and used to induce an antibody response. The first experiments using this approach look very promising, and this may become an important alternative to coupling with carrier proteins.

Peptides usually are synthesized with an automated machine using solid-phase techniques. The methods for synthesis and purification of the peptide are beyond the scope of this book. However, to judge the success of the coupling reaction and to determine the number of moles of peptide bound to the carrier, a small proportion of the peptide needs to be labeled. This can be done by including a small amount of $^{14}C$-labeled amino acid in the synthesis or by iodinating a sample of the peptides on a tyrosine or histidine residue (see p. 324) after the synthesis. A small sample of these iodinated peptides can then be added to the coupling reactions to ascertain the success of the coupling.

During immunization, antibodies to the carrier proteins or the coupling agent will also be produced, and these are normally removed by affinity-purifying the anti-peptide antibodies on a column prepared with conjugates of the peptide and a second carrier molecule. Techniques for affinity purification of the antibodies are described in general on p. 313.

### Designing the Peptide

Probably the most frequently asked question concerning synthetic peptides is what sequence should be used for the immunogen (reviewed in Doolittle 1986). Although there is no one correct answer, enough anti-peptide antibodies have been raised to make suggestions for peptide choices. However, preparing anti-peptide antibodies is still an empirical exercise. What works well for one immunogen may fail completely for another.

### Choosing the Appropriate Peptide Sequence

With careful synthesis, coupling, and immunizations, most sequences can be used to induce antibodies specific for the peptide itself. When considering which sequence to use, most people actually want to know how likely will it be that the anti-peptide antibodies will recognize the native protein. Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981, 1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria. In assessing the value of these criteria, hydrophilicity is required but is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies. Therefore, hydrophilicity can be thought of as required but not sufficient for choosing peptide sequences (see p. 661 for hydrophilicity values). Hydrophilic peptides are also more likely to be soluble for coupling reactions.

The presence of proline residues in synthetic peptides originally was suggested because $\beta$-turns often form portions of known epitopes. However, the presence of proline residues in peptides does not have much predictive value when antisera are tested for binding to the surface of native proteins. Although many excellent anti-peptide antisera have been prepared against sequences with proline residues, there is not sufficient evidence to target prolines when designing peptides.

More recently, several workers have noted that carboxy-terminal sequences often are exposed and can be targeted for anti-peptide sequences. Although using carboxy-terminal sequences does not guarantee that the resulting antibodies will recognize the native protein, a surprisingly high percentage will. Similarly, many amino-terminal regions are exposed, and these also may make good targets.

Another potentially useful parameter for selecting peptide sequences is the "mobility" of the amino acid residues. Originally, it was noted that the regions of a protein that become epitopes often have a higher temperature than other regions, as determined by NMR and X-ray structure (Moore and Williams 1980; Robinson et al. 1983; Tainer et al. 1984; Westhof et al. 1984). Higher temperature in crystallography and NMR distinguishes regions that are more mobile from

regions that are more static. These observations have led to the suggestion that stretches of amino acids that are more flexible are more likely to be epitopes. In the preparation of anti-peptide antibodies, when a peptide is coupled to a carrier molecule, it has a different local environment than in the original protein. When choosing a sequence for antibody production, a region of the protein that is more flexible will be more likely over time to form a structure that is similar to the peptide–carrier conjugate. Although the measure of mobility may become a useful criterion for selecting good peptide sequences, it has not been tested in enough detail to determine whether it will have any predictive value.

At present, a reasonable order of suggestions for choosing peptide sequences would be:

1. If possible, use more than one peptide.
2. Use the carboxyl-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the amino-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions; perhaps using longer peptides.

### *Size of the Peptide*

The smallest synthetic peptides that will consistently elicit antibodies that bind to the original protein are 6 residues in length. Responses to smaller peptides are typically weak or will not recognize the protein of interest, either in a native or denatured state. Since epitopes consisting of smaller regions have been reported, the lower limit presumably reflects the difficulty of recognizing the smaller peptides coupled to carriers. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and some will not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling.

In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. Two important preliminary questions to consider are: (1) Does the anti-peptide serum need to recognize the native protein? If so, use longer peptides or prepare anti-peptide antisera against multiple peptides. (2) How good is your peptide synthesis facility? Peptides over 20 residues in length are increasingly difficult to synthesize, yielding products with inappropriate side reactions. Longer peptides also are more likely to contain residues that make the coupling to carrier molecules more difficult. The correct decison between peptides with 10–15 residues and longer peptides will depend on the experimental design and will normally be a compromise between these factors. The safest choice, but also the most expensive, will be to prepare multiple small peptides of 10–15 amino acids in length from various regions of the sequence.

## Coupling Strategy

When choosing the sequence for a synthetic peptide, one factor that often is overlooked is the method of coupling. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic acid groups. Free amino groups used for coupling will be found on lysine side chains or on the amino-terminal residue. Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylic acid groups on aspartic acids, glutamic acids, and the carboxy-terminal residue. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino-terminal residue. When preparing antibodies against the carboxy-terminal region of the protein, the coupling should be done through the amino terminus of the peptide. Similarly, the coupling for amino terminal fragments should be done through the carboxy-terminal region of the peptide. For internal fragments, the major consideration is that the peptide be coupled by an end and not through a central residue.

The easiest strategy to manipulate the type of coupling is to add an extra amino acid on either the amino or carboxyl terminus to allow simple, one-site coupling to the carrier. Any coupling method that potentially can bind to an internal residue should be avoided. Similarly, coupling methods should be chosen that will bind to only one amino acid, if possible. If multiple coupling sites are possible, they should be localized to either the amino or carboxyl terminus, and the coupling should be adjusted to link only through one site per peptide on average. It is important to remember that it is often easier to use different peptides than design elaborate coupling schemes.

## Choosing the Appropriate Carrier

Many different carrier proteins can be used for coupling with synthetic peptides. The two most commonly used are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). Both work well in most cases, but each has disadvantages. Because of its large size, KLH is more likely to precipitate during cross-linking, and this can make handling KLH difficult in some cases. On the other hand, BSA is very soluble, but often is a good immunogen in its own right. For most purposes, either carrier will be adequate. Use whichever is more convenient.

Three other carriers that are used occasionally are ovalbumin, mouse serum albumin, or rabbit serum albumin. Ovalbumin can be used as a good carrier for most purposes. It is also a good choice for a second carrier when checking that antibodies are specific for the peptide itself and not the carrier. MSA or RSA may be used when the antibody response to the carrier molecule must be kept to a minimum.

BSA has 59 lysine (30–35 are available for coupling), 19 tyrosine, 35 cysteine, 39 aspartic acid, and 59 glutamic acid residues. Ovalbumin has 20 lysine, 10 tyrosine, 6 cysteine, 14 aspartic acid, and 33 glutamic acid residues.